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Differential expression of CD5 on B lymphocytes in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

CD5 is a cell surface molecule involved in antigen recognition and is present on all T lymphocytes and a subset of B lymphocytes. The purpose of this study was to examine CD5+ expression on peripheral blood B cells from healthy, noninfected cattle and cattle with subclinical and clinical paratuberculosis. Peripheral blood mononuclear cells (PBMC) were freshly isolated or cultured for 7 days in the presence or absence of live *Mycobacterium avium* subsp. *paratuberculosis* (*M. avium* subsp. *paratuberculosis*), and then analyzed by flow cytometry for CD5 expression within the B cell subpopulation. Analysis demonstrated a significant increase ($P < 0.01$) in B cells in clinical animals as compared to healthy control cows and subclinically infected cows. In addition, three subpopulations within the CD5+ B cell population were identified: CD5^{dim}, CD5^{bright}, and a minor population that was characterized as CD5$^{extra\ bright}$. A decrease in the CD5^{dim} B cell population along with a concomitant increase in CD5^{bright} B cells was observed in infected cows, an effect that was highly significant ($P < 0.01$) for subclinically infected cows in cultured PBMC. In vitro infection with live *M. avium* subsp. *paratuberculosis* did not affect CD5+ expression patterns on B cells, regardless of animal infection status. Addition of exogenous IL-10 to PBMC cultures resulted in decreased numbers of CD5^{bright} B cells for healthy control cows, whereas, a synergistic effect of IL-10 and infection with live *M. avium* subsp. *paratuberculosis* resulted in increased CD5^{bright} B cells for subclinically infected cows. These results suggest that differential expression of CD5^{bright} and CD5^{dim} subpopulations on B cells in animals with paratuberculosis may reflect a shift in host immunity during the disease process.

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1. Introduction

Paratuberculosis (Johne's disease) is an enteric disease of ruminants and is recognized as a worldwide problem in domesticated and wild species. Infection generally occurs in neonates after exposure to the intracellular pathogen, *Mycobacterium avium* subsp. *paratuberculosis*. Young animals can become infected with *M. avium* subsp. *para-tuberculosis* by ingestion of contaminated feces, colostrum, or milk or by in utero transmission from infected dams (Chiodini et al., 1984). During the early stages, the disease is characterized by a protracted period of subclinical infection during which a Th1-mediated immune response is dominant (Stabel, 2000). During this period animals are asymptomatic but will typically have elevated levels of pro-inflammatory cytokines such as IL-1, TNF-α, IFN-γ and IL-12 (Coussens et al., 2004). Upregulation of IFN-γ in the early stages of infection has been documented for other mycobacterial pathogens such as *M. tuberculosis* and *M. bovis* as well (Buddle et al., 2005; Dlugovitsky et al., 1997). The increase in IFN-γ is credited with controlling the progression of disease by augmenting T cell activation and

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T cell-macrophage interactions (Shankar et al., 2003). In the clinical stages of disease a shift to Th2-mediated immunity occurs with a concomitant decrease in Th1 responses. This shift in Th1–Th2 type immunity has been demonstrated in naturally and experimentally infected cattle and sheep with paratuberculosis (Stabel, 2000; Thorel et al., 1992).

Little is known about the role of specific T cell and B cell subsets in protective immunity to *M. avium* subsp. *paratuberculosis*. To date the majority of studies have shown that CD4+ T cells are highly activated during early infection and are the major source of IFN-γ. These observations have implicated CD4+ T cells as the key cell type involved in controlling the progression of the disease (Bassey and Collins, 1997). Less information is available about the function of B cells in paratuberculosis other than as sources of antigen-specific antibodies. B cells have been disregarded as integral players in the host immune response to *M. avium* subsp. *paratuberculosis* because the antibodies produced do not appear to be protective during the infectious process. However, in addition to the secretion of antibody, B cells play a role in the activation of CD4+ T cells. The decrease in CD4+ function noted in the latter stages of paratuberculosis may be closely associated with B cell activity. Previously, a significant increase in B cell numbers was noted in naturally infected cows demonstrating clinical signs of paratuberculosis as compared to subclinical cows or healthy controls (Waters et al., 1999). Interestingly, B cells isolated from the clinical cows did not proliferate in response to the *M. avium* subsp. *paratuberculosis* antigen preparation, whereas a normal proliferative response to the T cell mitogen, concanavalin A, was noted.

Characterization of B cell subpopulations present during the different stages of paratuberculosis may provide some insight into the progression of disease from a subclinical to a clinical state. CD5 is a membrane glycoprotein that is expressed on T cells as well as a subset of B lymphocytes, B-1a cells (Bondada et al., 2000). CD5 is an activation marker for B cells and is recognized as a mediator of T cell–B cell interactions (Werner-Favre et al., 1989). CD5+ B cells have been linked with autoimmune disorders such as lupus, rheumatoid arthritis, and multiple sclerosis through their production of polyclonal autoantibodies (Le Pottier et al., 2007; Polese et al., 2007). In addition, CD5+ cells produce natural antibodies to gut flora, suggesting that the reduced numbers of CD5+ cells observed in patients with chronic inflammatory bowel disorders (IBD) such as ulcerative colitis and Crohn's disease may exacerbate the progression of these inflammatory disorders (Neil et al., 1992; Polese et al., 2007). Interleukin-10 acts as an autocrine growth factor for the expansion of CD5+ B cells and plays a direct role in retarding the local inflammatory responses noted in enteric disorders, including paratuberculosis (Gary-Gouy et al., 2002; Khalifeh and Stabel, 2004a; Ramachandra et al., 1996).

Due to the chronic inflammatory nature of paratuberculosis and previous observations of B cell expansion in the peripheral blood of cows in the latter stages of disease, we hypothesized that the phenotype of the B cell population might be altered during advanced infection. With that in mind, we proposed to study the expression of the CD5 activation marker on B cells within the peripheral blood of dairy cows to assess the presence and stratification of CD5+ B-1a cells across the states of healthy, and subclinical, and clinical infection with *M. avium* subsp. *paratuberculosis*, with the hope that this information might prove useful in furthering our knowledge of host immune responses to this pathogen.

2. Materials and methods

2.1. Animals

The animals used in this study were randomly selected from a herd of Holstein dairy cows maintained on-site at the National Animal Disease Center (NADC), Ames, IA. The cows were placed in three groups based upon their infection status: (1) healthy noninfected controls, $n = 5$; (2) subclinical cows naturally infected with *M. avium* subsp. *paratuberculosis* but asymptomatic, $n = 5$, and (3) clinical cows naturally infected with *M. avium* subsp. *paratuberculosis* but demonstrating clinical signs of weight loss, $n = 6$. Groups of naturally infected cows and healthy control cows were housed separately on-site to prevent cross-contamination between groups of animals. Cows used in the study ranged in age from 3 to 6 years, regardless of infection status. Infection was monitored by bacteriologic culture for the fecal shedding of *M. avium* subsp. *paratuberculosis* by standard methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 CFU/tube (BBL™ Herrold's Egg Yolk Agar Slants with mycobactin J, amphotericin, nalidixic acid, and vancomycin; Becton Dickinson and Co., Sparks, MD) and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/tube and were asymptomatic. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3- to 5-year period and had previously been purchased from herds with no recent history of Johne's disease. In addition, these animals were negative on any serologic assays (i.e., production of antibody specific for *M. avium* subsp. *paratuberculosis* and IFN-γ) performed during that period. In contrast, subclinically infected cows had high antigen-specific IFN-γ responses and negligible Ab titers and clinically infected cows had low to moderate antigen-specific IFN-γ responses and high Ab titers. All animals were housed in BL-2 approved barns during the study and procedures utilized were approved by the Institutional Animal Care and Use Committee of the NADC prior to the initiation of the study. Animals were tested for Bovine Leukemia Virus (BLV) by an agar-gel immunodiffusion test using the BLV gp antigen as previously described (Miller and Van der Maaten, 1977).

2.2. Cell culture

Blood was collected from the jugular vein in 2× acid-citrate-dextrose (ACD) (1:10). Peripheral blood mononuclear cells (PBMC) were isolated from the buffy-coat fractions of the peripheral blood and resuspended in RPMI-1640 (Gibco, Grand Island, NY) containing 10% fetal calf

serum (Hyclone, Logan, UT), 100 units/ml penicillin G sodium (Gibco), 100 μg/ml streptomycin sulfate (Gibco), 0.25 μg/ml amphotericin B (Gibco), and 2 mM L-glutamine (Gibco). CD5 expression was evaluated within the B cell population of unfractionated PBMC on day 0 (freshly isolated) or after culture for 7 days. Unfractionated PBMC were plated at 2×10^6/ml in 1 ml volumes in 24-well flat-bottomed plates (Nunc Technologies, Rochester, NY) and cultured for 7 days at 39 °C in 5% CO_2 in a humidified atmosphere to allow development of monocyte-derived macrophages for phagocytosis of M. avium subsp. paratuberculosis. Additional control wells ($n = 2$) were set up for each animal to allow counting of adherent cells. On day 7 of the cell culture, nonadherent cells were removed from the wells and the wells were washed with cold $1\times$ PBS to remove the adherent cells from the plates. Adherent cells (80–90% monocyte-derived macrophages) were counted in 2 wells per animal prior to the addition of live M. avium subsp. paratuberculosis strain 19698 (NADC). Replicate wells ($n = 2$) containing unfractionated PBMC were then infected with live M. avium subsp. paratuberculosis at an MOI of 10 bacteria per adherent cell. After 24 h of infection, cells were harvested for flow cytometric analysis. Noninfected (media alone) control wells containing unfractionated PBMC but no M. avium subsp. paratuberculosis were maintained and harvested at this time point for comparison. Maintaining the total PBMC population in culture allowed cell–cell contact or crosstalk via antigen presentation and cytokine secretion to occur between macrophage, T and B cell populations, thereby simulating a more natural response to infection. The period of infection and dose of M. avium subsp. paratuberculosis for the in vitro experiments were chosen based upon previously published data and extensive experimentation within our laboratory (Khalifeh and Stabel, 2004b; Stabel, 1995).

Further experiments were designed to evaluate the effects of exogenous IL-10 on CD5 expression. Similar to above, cells were cultured in duplicate at 2×10^6/ml in 1 ml volumes in 24-well flat-bottomed plates (Nunc) at 39 °C in 5% CO_2 in a humidified atmosphere. Cells were stimulated with 100 ng/ml human IL-10 (Peprotech, Rocky Hill, NJ) or left without cytokine stimulation (control medium) for 18 h followed by infection with live M. avium subsp. paratuberculosis, strain 19698 at a ratio of 10 bacteria per cell. Cells were collected for flow cytometric analysis at 8, 24, and 72 h after cytokine treatment and in vitro infection with M. avium subsp. paratuberculosis. There is no commercial source for bovine IL-10 presently and the activity of this source of human IL-10 was successfully evaluated for cross-reactivity with bovine IL-10 antibodies in our laboratory (Khalifeh and Stabel, 2004b). The dose of exogenous IL-10 added to the wells and the time points evaluated were previously found to be effective at inducing changes in cellular activation as assessed by flow cytometric analyses, cytokine secretion, and phagocytosis of M. avium subsp. paratuberculosis (Khalifeh and Stabel, 2004b).

2.3. Bacteria

M. avium subsp. paratuberculosis strain 19698 was grown in Middlebrook 7H9 broth (pH 6.0) supplemented

with mycobactin J (Allied Monitor, Fayette, MO) (2 mg/l) and oleic acid-albumin-dextrose complex (Becton Dickinson Microbiology, Sparks, MD). The bacteria were harvested, washed three times with PBS (pH 7.4) (0.15 M), and resuspended in PBS to a final concentration of 10^9 ml^{-1} as determined by the absorbance at 540 nm. Bacterial stocks were then frozen in PBS at -80 °C until use in experiments. Prior to in vitro infection, frozen bacterial stocks were thawed, and clumps were dispersed by brief sonication at 25 W for 40 s with a Tekmar sonic disruptor (Tekmar, Lorton, VA). The viable cells in the frozen bacterial stocks were determined by culturing on HEYM. Viable cells in stocks were reduced by $1\log_{10}$ to approximately 10^8 CFU/ml after a freeze–thaw cycle so this value was used as the stock concentration to dilute the inocula.

2.4. Flow cytometric analyses

For analysis of the expression of CD5 on B cells, three-color flow cytometry protocol was used. Briefly, 100 μl of cell cultures at 2×10^6 cells/ml were incubated for 20 min at RT with monoclonal antibodies to B cell markers (VMRD, Pullman, WA) (7 μg/ml; BAQ155A, GB25A, LCT27A, and VPM30) and CD5 (VMRD) (7 μg/ml; B29A). A variety of B cell markers were evaluated initially in order to ascertain their abilities to stain B lymphocyte populations in cattle with paratuberculosis. Monoclonal antibody BAQ155A is a B lymphocyte marker for the bovine that labels surface IgM (VMRD). In contrast, monoclonal antibodies GB25A, LCT27A, and VPM30 react with B lymphocytes at specific stages of maturity or activation. GB25A recognizes the CD21 molecule, a maturation marker, and cross-reacts with a subset of B cells, follicular dendritic cells, and immature thymocytes; LCT27A reacts with the CD45R epitope on naïve B cells and T cells, and the VPM30 antibody reacts with B cells and activated T cells (Davis et al., 1995; Campbell et al., 1998). After labeling with the primary antibody, cell surface markers were then visualized by incubating cells for 20 min with 50 μl of phycoerythrin-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates, Inc., Birmingham, AL) (diluted 1:500 (v/v) to detect cells labeled with CD5 and 50 μl of PerCP-conjugated rat anti-mouse IgG$_1$ (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) diluted 1:25 (v/v) to detect cells labeled with anti-B cell monoclonal antibody. Cells were washed and resuspended in FACS buffer (PBS with 1% FCS and 0.01% sodium azide) containing DAPI stain at a 1:1000 dilution (10 mg/ml; Sigma, St. Louis, MO) in order to eliminate dead cells from the analysis. Cells were then washed and resuspended in FACS buffer and analyzed the same day by flow cytometry. Data from 5000 events that were DAPI negative (i.e., live cells) per sample were acquired using a FACSCAN flow cytometer (BD, LSR; Cell Quest software, BD Biosciences, San Jose, CA). Data were analyzed using Flowjo software (Tree Star, Inc., San Carlos, CA). Analysis was performed on replicate samples from each animal within the 3 treatment groups, healthy noninfected controls, subclinically infected, and clinically infected, with 5–6 animals per group.

2.5. Statistical analysis

Results were analyzed by using ANOVA, and significant differences between means were tested using Fisher protected least significant difference test using the Statview software package (Graphpad, San Diego, CA).

3. Results

3.1. Comparison of anti-CD5 antibodies

Four different bovine B cell monoclonal antibodies, BAQ155A, GB25A, LCT27A, and VPM30, were evaluated for use in this study. Results of staining freshly isolated PBMC from cows of different infection status demonstrated that clinically infected cows had significantly ($P < 0.01$) greater numbers of B cells compared to healthy noninfected control cows for all 4 antibodies (Fig. 1). Staining cells with either BAQ155A or GB25A monoclonal antibodies resulted in very distinct separation of the clinically infected cows from either subclinically infected or healthy controls. Alternatively, staining of unfractionated PBMC with either LCT27A or VPM30 antibodies failed to distinguish a difference in the percentages of B cells between subclinically and clinically infected cows although there was a significant ($P < 0.01$) difference in B cell numbers between the infected groups and the controls. It is possible that the VPM30 antibody, in particular, was co-staining a subset of activated T cells present in the PBMC isolated from subclinically infected cows, confounding the results as shown. Subsequent data presentation represents the sole use of the BAQ155A antibody (IgM+ B cells), although the profile of CD5+ expression on B cell populations stained with either the LCT27A or GB25A antibodies was similar to BAQ155A (data not shown).

3.2. Effect of infection status on CD5 staining

Animal infection status had a significant impact on the level of CD5 staining within the B cell population of freshly isolated cells (Fig. 2). Healthy cows tended to have higher ($P < 0.10$) percentages of CD5+ B cells as compared to subclinically infected cows. In contrast, the subclinically infected cows had a tendency for higher ($P < 0.08$) percentages of CD5- B cells as compared to control cows. Clinically infected cows tended to follow the same pattern as subclinically infected cows. Interestingly, we were able to discern 3 subpopulations of CD5+ B cells, extra bright, bright, and dim, by stratifying the CD5+ B cells according to staining intensity. Within those subpopulations, no significant differences were noted between treatment groups for CD5$^{extra\ bright}$ or CD5^{bright} staining so these subpopulations were collapsed into one and thereafter the 2 major subpopulations were identified as CD5^{bright} and CD5^{dim}. A reciprocal decline in the percentage of CD5^{dim} B cells was noted for infected cows compared to healthy controls (23.4 ± 5.6 and 25.4 ± 5.5 vs 38.7 ± 8.1, respectively), with mean differences approaching significance ($P = 0.11$) for healthy controls and subclincally infected cows. A FACS plot representing one individual animal from each infection group demonstrates the differences in CD5 staining intensity for the CD5^{dim} and CD5^{bright} populations (Fig. 3).

3.3. Effect of in vitro infection with *M. avium* subsp. *paratuberculosis* on CD5 staining

Further delineation of CD5 subpopulations was noted after PBMC were cultured for 7 days with and without live *M. avium* subsp. *paratuberculosis* (Fig. 4). The percentage of B cells in the total PBMC population was significantly ($P < 0.01$) lower for subclinically infected cows as compared to healthy controls and clinical cows (Fig. 4A). Although the percentage of B cells in the total cell population remained elevated for the clinically infected cows compared to the controls, mean values were not statistically different ($P = 0.13$). The percentage of B cells was not affected by in vitro infection with live *M. avium*
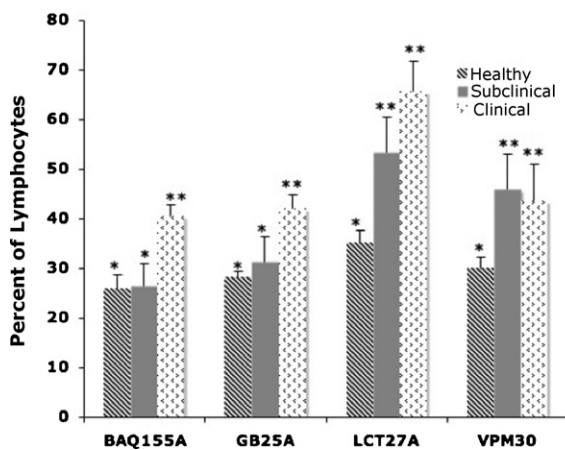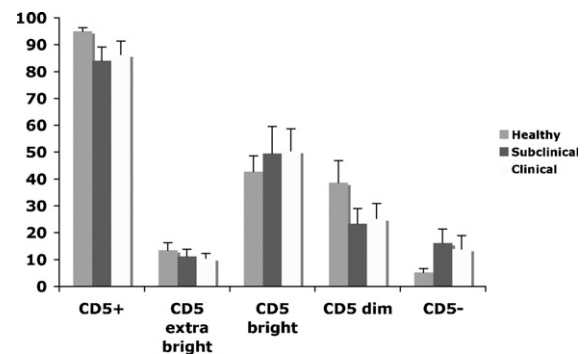

Fig. 1. Staining of B cell populations (% of total PBMC) in freshly isolated PBMC was compared using 4 different bovine B lymphocyte monoclonal antibodies, BAQ155A, GB25A, LCT27A, and VPM30. Treatment groups were: (1) healthy noninfected cows ($n = 5$); (2) subclinically infected cows ($n = 5$); and (3) clinically infected cows ($n = 6$). Data are presented as mean percentage of B cells $\pm$ S.E.M. for cows within each treatment group. Mean values with different symbols (*, **) are statistically different ($P < 0.05$) from other treatment groups within the same B cell marker.

Fig. 2. Different subpopulations of B cells (CD5+, CD5$^{extra\ bright}$, CD5^{bright}, CD5^{dim}, CD5) are delineated by the intensity of staining for the CD5 marker on freshly isolated PBMC obtained from: (1) healthy noninfected cows ($n = 5$); (2) subclinically infected cows ($n = 5$); and (3) clinically infected cows ($n = 6$). Data are presented as mean percentage of cells $\pm$ S.E.M.
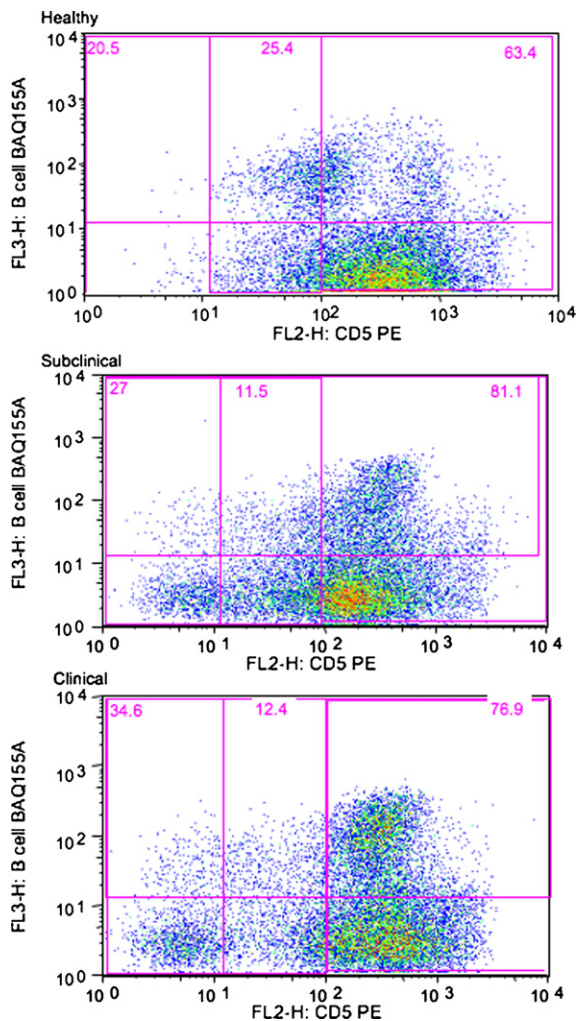
Fig. 3. A FACS plot demonstrating the differences in the intensity of CD5 staining on B cells from freshly isolated PBMC obtained from: (A) a healthy noninfected cow; (B) a subclinically infected cow; and (C) a clinically infected cow. Sectors 1 through 3 depict percentage of CD5- B cells, CD5^{dim} B cells, and CD5^{bright} B cells, respectively.

Fig. 4. Delineation of total B cells and CD5+ B cell subpopulations in 7 day PBMC isolated from: (1) healthy noninfected cows ($n = 5$); (2) subclinically infected cows ($n = 5$); and (3) clinically infected cows ($n = 6$). Unfractionated PBMC were cultured in complete medium for 7 days then infected with live *M. avium* subsp. *paratuberculosis* (MAP; 10:1 bacteria to adherent cell ratio) for an additional 24 h. Data are presented as mean percentage of cells $\pm$ S.E.M. Treatment groups with different symbols (*, **) are statistically different ($P < 0.05$) from one another within the medium alone or live *MAP* infection in vitro treatments.

subsp. *paratuberculosis*. The pattern of CD5^{dim} staining was similar to that noted previously for freshly isolated cells but more definitive, with lower ($P < 0.01$) numbers of B cells expressing CD5^{dim} in the cells isolated from infected cows relative to healthy noninfected control cows (Fig. 4B). The percentage of CD5^{bright} B cells was significantly ($P < 0.05$) higher for subclinically infected cows compared to the other 2 treatment groups (Fig. 4C). The CD5^{dim} B cell subpopulation was not affected by the addition of live *M. avium* subsp. *paratuberculosis* to the cell cultures within treatment groups, however, the percentage of CD5^{bright} B cells increased significantly ($P < 0.05$) after exposure of cells to the pathogen.

3.4. Addition of exogenous IL-10 and *M. avium* subsp. *paratuberculosis* on CD5

Stimulation of cultured PBMC with IL-10 and live *M. avium* subsp. *paratuberculosis* for 8, 24, and 72 h resulted in
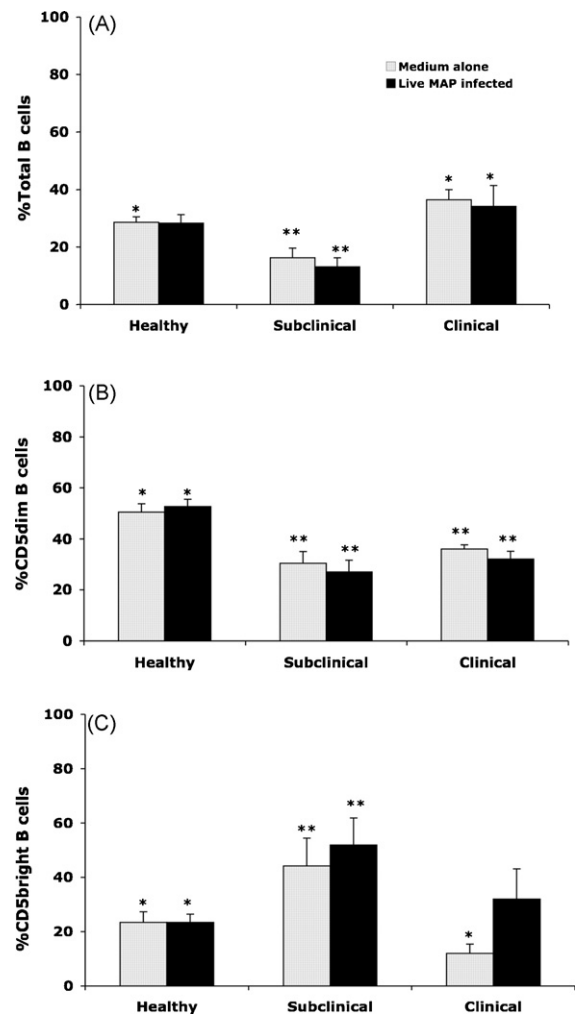
higher ($P < 0.01$) CD5^{bright} expression on B cells for infected cows, with greater expression for clinically infected cows ($P < 0.0001$) compared to healthy controls (Fig. 5). Culture of PBMC isolated from healthy cows with IL-10 for 8 h attenuated CD5^{bright} expression compared to cells incubated with medium alone (Fig. 5A). This pattern was observed regardless of the presence of live *M. avium* subsp. *paratuberculosis* in cultures. A similar trend toward lower CD5^{bright} expression on B cells was noted for clinical cows upon the addition of IL-10 to cell cultures, however, this effect was not maintained when cells were treated with IL-10 in the presence of live *M. avium* subsp. *paratuberculosis*. There was a significant effect ($P < 0.05$) of in vitro infection with live *M. avium* subsp. *paratuberculosis* on CD5^{bright} expression at 24 h for naturally infected
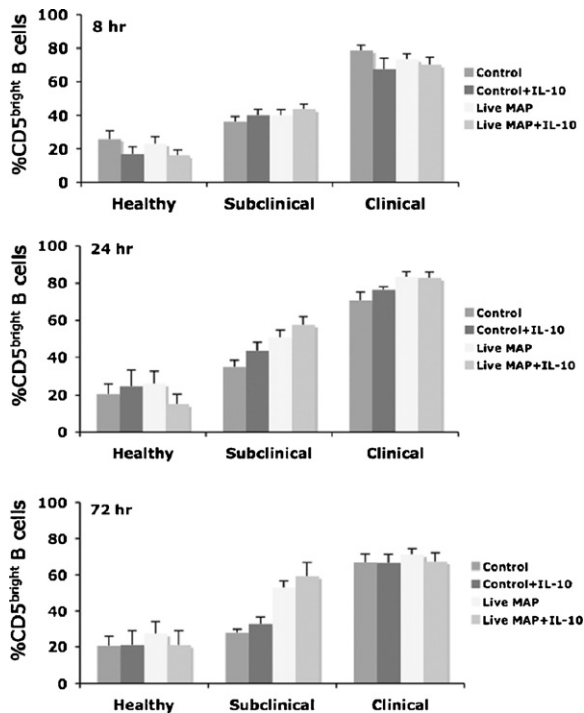
Fig. 5. Effects of recombinant IL-10 (100 ng/ml) and live *M. avium* subsp. *paratuberculosis* (MAP) infection on CD5^{bright} expression on B cells in PBMC isolated from: (1) healthy noninfected cows ($n = 5$); (2) subclinically infected cows ($n = 5$); and (3) clinically infected cows ($n = 6$). Cells were cultured for 8, 24, and 72 h. Data are presented as mean percentage of cells ± S.E.M. Overall effects: Time, 8 h vs 24 h, $P < 0.05$; Infection status, $P < 0.0001$; Live MAP infection, $P < 0.01$. At 8 h incubation: Infection status, $P < 0.01$. At 24 h incubation: Infection status, $P < 0.0001$; live MAP infection, $P < 0.001$; infection status × live MAP infection interaction, $P < 0.001$. At 72 h incubation: infection status, $P < 0.0001$; live MAP infection, $P < 0.01$; infection status × live MAP infection interaction, $P < 0.01$.

cows, an effect that was not noted for healthy control cows (Fig. 5B and C). In addition, a synergistic effect of IL-10 and *M. avium subsp. paratuberculosis* for cells from subclinically infected cows was observed at 24 h of incubation with higher ($P < 0.01$) CD5^{bright} expression on stimulated B cells ($57.6 ± 4.5$) as compared to control cultures with medium alone ($35.2 ± 3.2$) (Fig. 5B). In contrast, co-incubation of PBMC with IL-10 and live *M. avium subsp. paratuberculosis* for 24 h resulted in continued attenuation of CD5^{bright} expression on B cells for healthy cows. After 72 h of incubation, infection of cells with live *M. avium* subsp. *paratuberculosis* invoked significantly ($P < 0.01$) greater CD5^{bright} expression on B cells with no further increases noted due to the addition of IL-10 (Fig. 5C). This effect was noted only for subclinically infected cows.

3.5. Bovine leukemia virus status of cows and CD5 expression

In the present study, healthy control cows were negative for BLV but several cows with paratuberculosis were also positive for BLV. Six of the 11 paratuberculosis cows were positive for BLV with the numbers randomized equally between the subclinical and clinical infection

groups. However, BLV status did not significantly alter the CD5^{bright} or CD5^{dim} subpopulations of B cells (data not shown). Further validation that the shift in the pattern of CD5 staining in naturally infected cows was not influenced by BLV status was ascertained by splitting out CD5 expression data after in vitro infection with *M. avium* subsp. *paratuberculosis*. The addition of live *M. avium* subsp. *paratuberculosis* to cell cultures shifted the CD5+, CD5−, and CD5^{dim} populations to mirror the expression patterns observed previously for naturally infected cows that included both BLV positive and negative cows (data not shown).

4. Discussion

Gaining further insight into host immune responses to *M. avium* subsp. *paratuberculosis* infection is critical to the control and management of this disease. Similar to other mycobacterial diseases, Th1-mediated immunity plays a significant role in controlling *M. avium* subsp. *paratuberculosis* infection, yet many aspects of immunity contribute to the regulation of the infection within the host. Our current understanding suggests that Th2-mediated immunity is not protective and cannot control mycobacterial infections. This assumption arises from observations that Th1 immunity switches to Th2-mediated immunity as paratuberculosis progresses from the asymptomatic to the more clinical state, and that Th2 becomes the dominant host response in clinical disease. Once the animal succumbs to a clinical state it is assumed that immunologic control of the pathogen is lost. However, little information is available to formulate a hypothesis for why this occurs. The principal role of T cells in the control of mycobacterial infections, including *M. avium* subsp. *paratuberculosis* has been well described (Koo et al., 2004; Waters et al., 2003), yet there is a lack of data on B cells.

Commonly, B cells are key components of the adaptive immune system, providing immune surveillance through the production of antibodies for the neutralization of predatory pathogens. B cells also provide accessory help to T cells through CD40 signaling, resulting in the upregulation of co-stimulatory molecules CD80 and CD86, as well as adhesion molecules, further enhancing activation of T cells (Bishop and Hostager, 2003). The segregation of B cell populations may be accomplished by using activation markers such as CD5 to separate them into B-1 (CD5+) and B-2 (CD5−) classes. B-2 cells represent the normal circulating B cell population (Le Pottier et al., 2007). The B-1 population can be further subdivided into B-1a and B-1b subclasses. Although both subclasses are CD5+, B-1b cells lack the surface receptor for CD5 so B-1a cells represent the CD5+ B population described here (Youinou et al., 1999). In the bovine, the CD5+ T cell populations can be further differentiated by the intensity of staining into either "bright" or "dim" subsets (O'Reilly and Splitter, 1990). CD5^{bright} cells are CD2 positive and either CD4 or CD8, whereas CD5^{dim} T cells poorly express these receptors and are highly positive for γδ TCR (O'Reilly and Splitter, 1990).

It is not clear what the bright and dim delineation within the CD5+ population represents on a functional

basis for B cells, however, one study showed that less than 13% of CD5^{dim} cells were IgM positive (O'Reilly and Splitter, 1990), suggesting that the majority of B-1a cells would be CD5^{bright}. In the present study, we were able to demonstrate a clear distinction between subpopulations of CD5+ B cells based upon the staining intensity with 50–60% of the total CD5+ population identified as CD5^{bright}. The percentage of cells stratified into CD5^{bright} and CD5^{dim} subsets was influenced by the infection status of the cows, with CD5^{dim} B cells reduced 35–40% for cows with paratuberculosis compared to healthy controls. The reduction in the CD5^{dim} population was concurrent with decreased numbers of CD5+ cells for both subclinical and clinical infection groups. Human patients with chronic inflammatory bowel disease such as Crohn's disease and ulcerative colitis have reported reduced numbers of circulating CD5+ B cells (Neil et al., 1992; Polese et al., 2007). One possible mechanism for the decrease in peripheral blood CD5+ B cells in patients with inflammatory bowel disease is the trafficking of these cells into the gut (Polese et al., 2007). This was suggested since B-1a cells are polyclonal reactors that are critical in controlling luminal antigens. The chronic inflammatory pathology of the terminal ileum suggests that such a mechanism may be consistent for cattle with paratuberculosis as well. An influx of cells into the intestinal tissue occurs during the progression of paratuberculosis with documented increases in the number of macrophages, CD8+ and $\gamma\delta$ T cells (Little et al., 1996; Navarro et al., 1998; Lee et al., 2001), and decreased numbers of CD4+ cells (Koets et al., 2002). Very few studies have reported data on the effects of disease status on B lymphocyte numbers in the affected tissues. One report describing early *M. avium* subsp. *paratuberculosis* infection demonstrated that the T/B cell ratio was increased in infected lambs compared to noninfected controls (Begara-McGorum et al., 1998), however, no data are available documenting changes in the T/B ratio in tissues during the progression from an asymptomatic to a more clinical state of disease.

The shift from CD5^{dim} to CD5^{bright} B-1a cells may suggest a regulatory mechanism to protect against chronic inflammatory events. Studies on inflammatory bowel disorders such as Crohn's disease and ulcerative colitis suggest that B cells may provide a protective role (Mizoguchi et al., 2002; Wei et al., 2005), with a suggested mechanism being enhanced surveillance of enteric bacteria through interactions with T regulatory cells and increased expression of CD1d and IL-10 (Mizoguchi et al., 2002; Wei et al., 2005). Interestingly, human peripheral blood CD5+ B cells can be induced to produce IL-10 upon activation by the B cell receptor (Gary-Gouy et al., 2002). An upregulation of IL-10 expression by monocytes or PBMC from subclinically infected cows has been reported after exposure of cells to live *M. avium* subsp. *paratuberculosis*, and increased IL-10 expression has also been observed in the ileum and associated lymph nodes for clinically infected cows (Coussens et al., 2004; Khalifeh and Stabel, 2004b; Weiss et al., 2005). In the present study, IL-10 secretion was greater for unfractionated PBMC isolated from infected cows compared to healthy controls (data not shown), however, due to limitations of our laboratory we were unable to assess IL-10 secretion by the CD5-specific B cell subpopulations. Yet the addition of exogenous IL-10 to PBMC cultures in concert with live *M. avium* subsp. *paratuberculosis* resulted in greater percentages of CD5^{bright} B cells, suggesting that IL-10 may be involved in the regulation of the B-1 cell subpopulation. This observation is important as evidence suggests that IL-10 acts as an autocrine growth factor for the expansion of B-1 cells (Gary-Gouy et al., 2002; Ramachandra et al., 1996).

Alternatively, the shift from CD5+ to CD5− cells noted between infection groups could be reflective of a change in cell phenotype. The discrimination of CD5+ cells into CD5^{dim} and CD5^{bright} subpopulations did present some interesting observations for the naturally infected cows. The overall increase in total B cell numbers was in conflict with the reduced numbers of CD5+ cells observed in clinically infected cows. Increased numbers of peripheral blood B cells in clinical disease is an observation that has been highly repeatable in our laboratory (Waters et al., 1999). In a previous study we had discounted BLV as a causative factor for the increased percentage of B cells but subclinical BLV has a high prevalence in the US, infecting up to 89% of dairy herds (USDA, APHIS, VS, 1997). A hallmark characteristic of BLV PL+ (persistently lymphocytotic) cattle is increased numbers of B cells in the peripheral blood but, unlike the B cell population for the clinically infected cows in this study, the majority of these B cells are typically CD5+ (Depelchin et al., 1989; Williams et al., 1988). Stratification of cows in the present study by BLV status did not influence the pattern of CD5 staining on the B cell population noted for subclinical and clinical cows. Since clinical paratuberculosis is associated with increased secretion of antigen-specific antibodies, it seems likely that the expanded population of B cells observed in clinically infected cows consisted of B-2 lymphocytes.

Further, the dichotomous shifts from CD5^{dim} to CD5^{bright} within the CD5+ B cell population for subclinically infected cows and the expansion of B cells in clinically infected cows may represent 2 distinct populations of activated B cells. Haas and Estes (2000) demonstrated the expansion of CD5+ B cells upon cross-linking of cells with the B cell receptor (sIgM) and suggest that this would occur upon exposure to various pathogens. Alternatively, their work further showed that B cells activated through CD40 signaling would inhibit expression of the B-1 type phenotype yet cells would remain highly proliferative. In the present study, it is conceivable that subclinically infected cows are able to control and manage the inflammatory responses due to *M. avium* subsp. *paratuberculosis* through the expansion of CD5+ B-1a cells that would be followed by a consequent increase in T regulatory cells. In contrast, the B cell population from clinically infected cows may be skewed away from B-1a cells toward a B-2 subpopulation, as suggested by the production of nonprotective antibodies observed during clinical disease. Further analysis of the B cell subpopulations and the interplay between CD5 expression and IL-10 secretion during the different stages of infection is warranted in order to gain greater understanding of potential mechanisms involved in the control of infection by the host.

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5. Conclusions

The shift in CD5 expression on B cells in unfractionated PBMC (dim to bright) in naturally infected cattle may be representative of changes in the B cell subpopulations, an observation that is further supported by increases in CD5^{bright} B cells upon the addition of IL-10 to cultures. In addition, this shift may be representative of a change in B cell subpopulation from the typical B-1a to a B-2 population, particularly for clinically infected cows. These observations are important pieces of information that will ultimately help us learn more about T cell–B cell interactions during *M. avium* subsp. *paratuberculosis* infections and how these interactions align with sub-clinical and clinical disease states.

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